

PROTEIN CRYSTAL GROWTH IN A MICROGRAVITY ENVIRONMENT

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Note: This report is based primarily on the contents of two recent publications describing the background and results of the space shuttle experiments:

1. Charles E. Bugg
"The Future of Protein Crystal Growth", Journal of Crystal Growth 76 (1986) 535-544.
2. Lawrence J. DeLucas, F. L. Suddath, Robert Snyder, Robert Naumann, M. Beth Broom, Marc Pusey, Vaughn Yost, Blair Herren, Daniel Carter, Bill Nelson, Edward J. Meehan, A. McPherson and C. E. Bugg
"Preliminary Investigations of Protein Crystal Growth Using the Space Shuttle", Journal of Crystal Growth 76 (1986) 681-693.

I. Introduction

Crystallography is a powerful method for determining the three-dimensional structures of complicated biological molecules. Crystallographic studies of proteins and nucleic acids have played key roles in establishing the structural foundations of molecular biology and biochemistry, and for revealing structure/function relationships that are of major importance in understanding how other

macromolecules operate in biological systems. More recently, crystallographic studies of biological macromolecules have become of considerable interest to the pharmaceutical, biotechnology and chemical industries, as promising tools in protein engineering, drug design, and other applications to biological systems.

The elaborate information that can be learned from the three-dimensional structure of a protein is useful in a variety of ways. From the basic biological viewpoint, this information underlies our current understanding of the mechanisms by which enzymes, receptors, hormones, etc. function in biological systems. Within the pharmaceutical industry, protein structure information can be helpful in the development of novel drugs. Since many pharmaceutical agents exercise their activity by interacting with proteins, knowledge of the three-dimensional structure of a target protein can be used to design compounds that selectively bind to protein sites and thereby inhibit the activities of the protein (1-5). Several major pharmaceutical companies have now established protein crystallography groups to pursue this approach of rational drug design.

Another highly promising application of protein crystallography is in protein engineering (6-15). Readily available techniques of molecular biology permit investigators to specifically alter protein molecules by site-directed mutagenesis. In general, the most promising approaches to protein engineering depend upon detailed structural information about the proteins of interest. Although the techniques that are involved in protein engineering are under intense development at this stage, it is generally accepted that these methods will prove to be of tremendous practical value for the design of modified enzymes, and for the development of proteins that have carefully engineered physical and biological properties. There is likely to be a continued expansion of interest in applications of site-directed mutagenesis, and several protein crystallography groups in academic institutions and in biotechnology-oriented companies are expanding their programs in order to exploit the potential of this technology.

An additional use of protein crystallography is in the design of synthetic vaccines (16-19). Several recent studies have indicated that effective vaccines might be made from synthetic peptides that are representative of protein segments found on the surfaces of target proteins. Protein crystallography provides one of the most effective techniques for locating those peptides.

Because of the widespread fundamental and practical importance of knowing the structures of biological materials, the overall interest in protein crystallography has increased greatly during the past few years. At the present time, protein crystallography really has no substitute: It is the only general technique available for elucidating the precise atomic arrangements within complicated biological molecules. Other techniques, such as two-dimensional NMR spectroscopy, are likely to become more and more useful during the next few years, but it is unlikely that any of these techniques will be competitive with X-ray crystallography in the near future for routinely determining three-dimensional structures of large proteins and other complicated biological macromolecules. Unfortunately, protein crystallography has the unique requirement that relatively large, high-quality single crystals must be obtained before a structural study can be pursued. Therefore, protein crystal growth has become a topic of considerable importance.

Unfortunately, all of the major steps that are involved in determining a protein structure by crystallographic techniques are subject to a number of experimental difficulties. Most of the proteins that have been studied during the past three decades required many years of intense effort before the complete three-dimensional structure was known. Consequently, until recently, there was limited interest in using protein crystallography as a general tool in biological research, and protein crystallography programs were limited to a few laboratories, primarily in academic institutions.

Several recent advances in the technologies required for protein crystallographic studies have made it much easier to determine the crystal structure of a protein or other macromolecule:

(1) Recombinant DNA techniques have made it possible to obtain proteins that would have been impossible to isolate in sufficient quantities for X-ray diffraction studies several years ago (6-13).

(2) Synchrotron radiation sources, which provide X-ray beams with intensities that are several orders of magnitude greater than those available from laboratory sources, permit data sets to be collected rapidly, and from relatively few crystals (22-27). Synchrotrons also offer the capability of selecting specific wavelengths, and this capability might eventually prove to be of value in eliminating the need for heavy-atom derivatives in some specific cases (26).

(3) Electronic area detector systems have been developed, and are now available commercially from several different vendors. These two-dimensional detector systems permit data to be measured much faster than was possible with the single-counter detector systems that have been widely used in the past (28, 29).

(4) Recent applications of anomalous dispersion measurements have permitted protein structures to be determined without the addition of heavy-atom derivatives (30-32). Intense efforts are in progress in a number of laboratories to develop direct methods for determining protein phases, which are analogous to the powerful statistical techniques that have essentially eliminated the need for heavy-atoms in "small molecule" crystallography (33-39).

(5) Computer graphics methods have revolutionized techniques that are used to construct protein models from electron density maps (40, 41). Computer graphics techniques have also made it possible to understand and to interpret structures with ease (41, 42), and the widespread use of computer graphics in the pharmaceutical, chemical and biotechnology industries has served to further stimulate interest in applications of protein crystallography.

(6) A variety of approaches and software systems have been developed for refining protein structures, once an initial model is constructed from the electron density maps (43-46). Refinements are now being pursued routinely to high resolutions, and the increasing availability of super computers will make refinements of large proteins relatively routine (47).

It is noteworthy that the major advances in protein crystallography involve those experimental steps that are of importance after suitable crystals have been obtained. For the most part, the general procedures that are used for growing protein crystals have not changed appreciably during the past few years. Most protein crystals are still grown by brute-force, trial-and-error methods, which require investigations of large numbers of experimental conditions in hopes of finding the combination that produces usable crystals. However, once good crystals of a particular protein have been obtained, the crystallography often moves along at a rapid rate.

2. Current problems in protein crystal growth

Nearly any protein crystallographer would immediately point to protein crystal growth as being the major bottleneck in the further widespread development in this field. Despite intensive efforts, a number of interesting proteins have never been crystallized. Many proteins and other biological macromolecules may yield small micro-crystals readily, but it can then take several years of tedious trial-and-error experimentation before these micro-crystals can be induced to grow large enough for a complete structural analysis. Even when large crystals are obtained, the crystals of essentially all biological macromolecules diffract very poorly, due to various types of internal disorder within these crystals.

Most protein crystallography laboratories could cite structural studies that have been delayed for years by problems with protein crystal growth. For example, the relative importance of the difficulties encountered with growing crystals that are

suitable for complete structural studies can be seen from our experiences in Birmingham with the enzyme human purine nucleoside phosphorylase (PNP), which is a trimeric protein with a molecular weight of about 97,000. We began our efforts to crystallize PNP in 1975, using enzyme isolated from several different sources. These efforts continued until 1981, when the first usable crystals of human PNP were grown (48). At the present time (early 1987), we have complete data sets collected for about one dozen substrate, substrate analog, and inhibitor complexes of PNP; we have refined the structure at 3.2 Å resolution (49); and we are well along with the 2.7 Å refinement of the structure. We had the benefit of most modern developments in protein crystallography, including map modification techniques, synchrotron data, molecular graphics for constructing and interpreting models, and automated refinement by restrained least-squares. The main point behind this history is that it took about twice as long to get usable crystals as it did to do the rest of the crystallography. Once we had suitable crystals, the project advanced fairly rapidly. Clearly, crystal growth was the major obstacle in the development of this research project. Even the best crystals that we now have are not highly ordered, which causes problems in efforts to refine the structure of the protein.

3. Microgravity applications to protein crystal growth studies

One interesting new development in protein crystal growth involves studies of crystal growth under the microgravity conditions that are available in space. Crystal growth has been of considerable interest to NASA and to other space-oriented researchers for a number of years (66), and various fundamental studies of crystal growth in space are in progress. The major motivation behind undertaking these space experiments is to examine the effects that density-driven convective flow has on crystal growth. In the absence of gravity, these density-driven convective flow effects are eliminated, thus permitting the role of convection on crystal growth

to be examined directly. In addition to the possibility of suppressing convective flow effects, microgravity conditions may also serve to minimize sedimentation, which can interfere with uniform growth of protein crystals. Another potential advantage of microgravity is the option of doing containerless crystal growth. Contacts with vessel walls can lead to heterogeneous nucleation. In the absence of gravity, it is possible to form stable spherical droplets of crystallizing materials, which may be suspended by acoustical levitation, air streams or other methods. It is also possible to form relatively large, stable droplets of aqueous solutions by simply extruding the solutions from a pipette or a syringe; thus protein crystals might be grown in relatively large droplets adhering to syringe tips, without the extensive wall effects that generally accompany crystallization experiments on earth.

Several projects to study protein crystal growth in space are now in progress (50-54). In addition, investigators at the Marshall Space Flight Center and other researchers supported by NASA, who have considerable background experience in fluid mechanics and crystal growth theory, are now turning their attention and diverse disciplines to fundamental investigations of protein crystal growth. Although it is not clear at this stage if microgravity conditions will prove of general importance in significantly enhancing the growth of protein crystals, the space experiments should make it possible to control parameters that would be difficult or impossible to control on earth. Thus these space experiments should prove useful in better understanding the principles, and the limiting factors in protein crystal growth.

NASA-sponsored space shuttle experiments in protein crystal growth

The space shuttle experiments have been described in considerable detail in the literature (53,54).

One of the most widely used methods of crystallizing proteins on earth involves the slow precipitation of protein from droplets of solution by vapor pressure equilibration against a solution containing a higher concentration of the

precipitating agent. The "hanging-drop method" is a common version of this general technique. Most protein crystallography laboratories have extensive experience with vapor-diffusion crystallization methods, and a large percentage of the protein crystals described in recent publications have been obtained by these methods.

In order to evaluate the possible effects of microgravity on protein crystal growth, it will be necessary to investigate a range of different types of proteins that have been well-characterized in ground-based studies. Many of the proteins that are being extensively studied in crystallography laboratories around the world are only available in milligram quantities. Vapor diffusion techniques are particularly suitable for crystallization experiments involving small quantities of protein and can be used for most types of biological materials. Therefore, we focused our initial experiments on development of a microgravity-adapted version of the popular hanging-drop method.

Prototype flight hardware for growing protein crystals by vapor-diffusion methods was developed, tested, and improved through a series of four shuttle flights in 1985 and 1986 (STS-51D, STS-51F, STS-61B and STS-61C). These experiments were performed in the middeck area of the shuttle. Although the prototype hardware was expected to be useful for testing concepts and developing the basic techniques for protein crystal growth in space, it was clear that it would be of limited value for performing systematic experiments in protein crystal growth, since relatively few samples could be included and important variables such as temperature could not be controlled.

The shuttle experiments were especially useful for optimizing the major variables in vapor diffusion protein crystal growth. We performed various experiments related to drop stability and found that large droplets (30-80 μ l) are stable on blunt syringe tips even when maneuvering rockets were fired while in orbit. Polypropylene syringes with flared tips, and blunt end glass syringe tips in several different diameters, with and without various types of coatings, have now been used successfully for retaining droplets of protein solutions. Although protein

crystals have been grown in droplets as large as 80 μ l, the experiments to date indicate that smaller droplets will assure complete equilibration during the limited period (3-6 days) available for protein crystal growth on space shuttle missions. In the most recent set of shuttle experiments (on STS-61C), 30 and 40 μ l droplets were used with good results. Ground-based and flight experiments also have provided qualitative information about equilibration rates within the vapor diffusion chambers. These studies have suggested that equilibration rates are significantly slower under microgravity conditions, presumably because of suppressed convection effects. The vapor equilibration chambers have now been designed to accelerate these equilibration rates. In addition, a reliable technique for seeding droplets of protein solutions within the vapor diffusion apparatus has been developed, and has been used to grow crystals of human C-reactive protein.

During the most recent shuttle experiments on STS-61C, crystals were grown of all proteins that were tested, including hen egg white lysozyme, human serum albumin, human C-reactive protein, bacterial purine nucleoside phosphorylase, canavalin, and concanavalin B. That particular shuttle mission was prematurely shortened, and the protein crystal growth experiments were deactivated during the third day of the flight. Although many of the protein solutions had not completely equilibrated during that period of time, relatively large X-ray quality crystals were obtained for all of the proteins except lysozyme.

Comprehensive studies of the effects of microgravity on protein crystal growth will be made on future shuttle flights, using equipment based on the prototype system that was flown on STS-61C. Although quantitative conclusions about protein crystal growth cannot be made at this stage, there are several interesting qualitative observations that have been made during these preliminary shuttle experiments.

It appears that the elimination of density-driven sedimentation can affect crystal morphology. The best example of this is canavalin, which grew crystals that were dispersed through the droplets in space. Nearly all the space-grown canavalin

crystals appear to have formed from separate nucleation sites, resulting in uniform morphologies. On the other hand, canavalin crystals grown by this method on earth generally form as fused aggregates at the bottom of the droplets. In the case of human C-reactive protein, an entirely new crystal form, which had not previously been identified in ground-based crystal growth experiments, was obtained from shuttle experiments. Crystallization of C-reactive protein has been studied extensively over the past eight years in Birmingham and only one crystal form, with space group $P4_122$ (or $P4_322$) has been obtained in these experiments. A new crystal form was first observed for C-reactive protein from experiments on STS-61B, and copious quantities of this crystal form were obtained on STS-61C. The space group for the new crystal form is $P4_222$, and it diffracts to an appreciably higher resolution than the original crystal form. The new crystal form has now been obtained in ground-based experiments using the shuttle hardware, so it may be influenced by altered equilibration rates or other experimental conditions that are hardware dependent. It is not yet clear how microgravity affects the distribution of these two crystal forms of human C-reactive protein.

It is not yet clear if the internal order or diffraction resolutions of space-grown protein crystals are significantly different from that of crystals grown on earth. It will be necessary to do detailed comparisons involving large numbers of crystals grown under well-controlled conditions on earth and in space, before the potential effects of microgravity on protein crystal quality can be evaluated.

Summary

Protein crystal growth is a major experimental problem and is the bottleneck in widespread applications of protein crystallography. Research efforts now being pursued and sponsored by NASA are making fundamental contributions to our understanding of the science of protein crystal growth. Microgravity environments offer the possibility of performing new types of experiments that may produce a better understanding of protein crystal growth processes and may permit growth environments that are more favorable for obtaining high-quality protein crystals.

A series of protein crystal growth experiments using the space shuttle has been initiated. The first phase of these experiments has been focused on the development of micro-methods for protein crystal growth by vapor diffusion techniques, using a space version of the hanging-drop method. The preliminary space experiments have been used to evolve prototype hardware that will form the basis for a more advanced system that can be used to evaluate effects of gravity on protein crystal growth.

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